STAIN TECHNOLOGY

VOLUME VIII APRIL, 1933 NUMBER 2

THE HISTORY OF STAINING

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JOSEPH VON GERLACH

It is quite customary to regard the originator of the modern staining technic as being Professor Joseph von Gerlach, located for many years in Erlangen, Germany. So important, in fact, were his contributions to this field that it was at one time commonly thought that he was the first to use any dye in histology—altho it is now known that several others had preceded him.

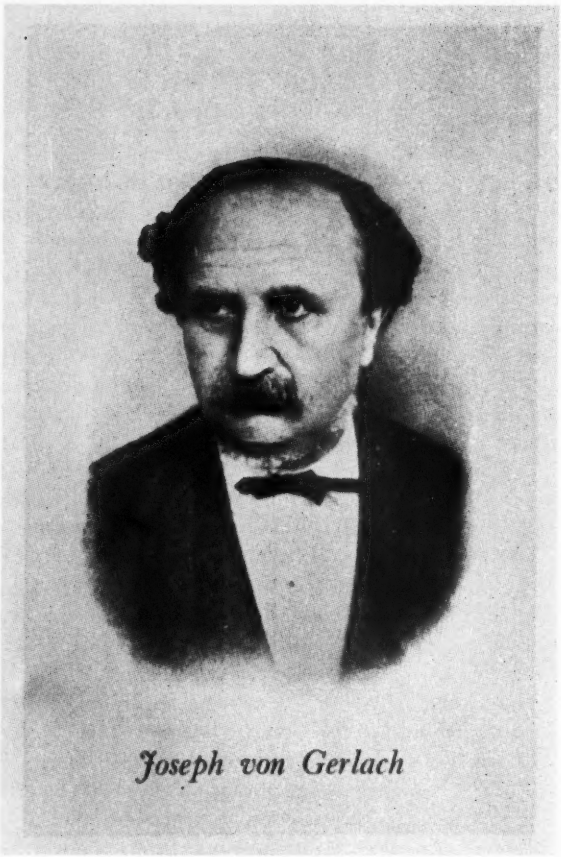
Gerlach was born in Mainz on April 3, 1820, and pursued his higher education during the years 1837 to 1841 at Würzburg, then Munich, and later Berlin. He received his doctor's degree in 1841 and spent the two following years traveling and visiting institutions at Paris, Vienna and London. In 1843 he returned to his birthplace and practised medicine there for six years. During this period he conducted research work in anatomy and microscopy and in 1848 published his famous "Handbuch der allgemeine und specielle Gewebelehre."

In 1850 he was called to the University of Erlangen where he spent the rest of his active life. He was Professor of Physiology there until 1872 and then in charge of Pathological Anatomy until 1865. From that time on his title was Professor of Anatomy. In 1891, just 50 years after receiving his doctor's degree, he celebrated his "Doctor's Jubilee" and at the same time retired from active connection with the university. He died five years later, December 17, 1896.

Thruout nearly the whole of his 50 years of active life he was one of the leading anatomists of Europe and contributed much to the development of microscopic technic and anatomy. The importance of his work in developing the technic of staining is nowhere better expressed than by Gierke,[1] a translation of whose comments follows:

Quite commonly Gerlach, the anatomist of Erlangen, is regarded as the founder of microscopical staining, because with his discovery of the staining ability of carmin and with his employment of this substance as a microscopic reagent, he interested histologists to use it

[1]Gierke, H. Farberei zu mikroskopischen Zwecken. Zts. Wis. Mikr., 1, 62–100. 1884. See p. 68.

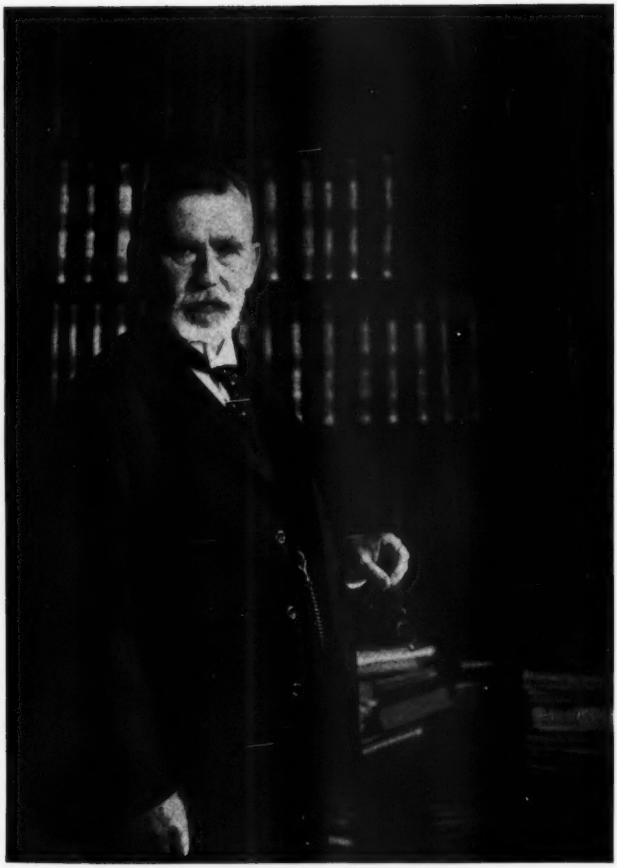


JOSEPH VON GERLACH
1820–1896

according to his method and to make further investigations with it and with other dyes. Gerlach must certainly be considered the founder of staining technic since histologists and zoologists employed this method on his recommendation and because the technic was given widespread employment after the publication of the paper just cited. He cannot, however, under any circumstance be considered the discoverer of the use of carmin and, therefore, of the technic of staining. Far from wishing, nevertheless, to discredit his great service to the technic in question, I must insist that he was in no way the first to employ and recommend carmin for microscopic purposes.

Gerlach's real contribution came, as is so often the case, partly from a fortunate accident and even more from his ability to appreciate the importance of that accident. Altho, as stated by Gierke in his above translation, he was not the first to employ carmin for microscopic purposes, he does seem to have begun its use independent of previous workers in this field. In 1854 he observed that tissues became colored after injection with a carmin gelatin. This led him to attempt staining of sections employing ammoniacal carmin, the solution which was then most commonly used, but for a long time without satisfactory results. The fortunate accident was when, in 1858, he left a cerebellum section, hardened in potassium bichromate, in a very dilute solution of ammonia carmin over night, thanks to an oversight on his part. When he found this section the next day it was beautifully stained with fine differentiation of nerve fibers and nerve cells. This showed him that previous failures had been due to the use of a too concentrated use of the dye.

Gerlach was very quick to see the significance of this accident and the importance of the technic which it suggested to him. He developed the methods to such an extent, and was so enthusiastic in teaching them to his students and in recommending them to other histologists, that in spite of the earlier use of carmin by such men as Hill, Göppert and Cohn, Ehrenberg, and Hartig, he is ordinarily considered—and undoubtedly correctly—the father of the staining method in histology.



PAUL EHRLICH
1854–1915

PAUL EHRLICH

Paul Ehrlich, who was born in Silesia in March, 1854, was one of the most striking scientific figures at the turn of the century and made important contributions to so many biological fields that in reviewing his life it is easy to overlook some of them. His contributions to bacteriology and to medicine have been told time and time again. That he also made equally important contributions to the technic and theory of staining is not so generally realized.

Ehrlich had a very broad medical training in that he attended four different medical schools—Breslau, Strasbourg, Freiberg and Leipzig. Always a non-conformist, he does not seem to have fitted into the accepted scheme of things at any of these institutions. He was after facts rather than university credit and he hardly distinguished himself according to the usual standards by which medical students were judged.

Ehrlich was associated with Koch (ten years his senior) in Berlin when the latter was doing his classical work on the tubercle organism. Koch had already shown the value of anilin dyes in staining bacteria. Ehrlich now discovered that they could be used to stain bacteria in the tissues, and in this way demonstrated the tubercle organism *in situ*. In doing this he showed the ability of this organism to resist the decolorizing action of acid, altho the recognition of the diagnostic value of this fact did not come until later.

Perhaps Ehrlich was somewhat too enthusiastic a student of tuberculosis, for he contracted the disease himself and might well have become one of the martyrs to science, of which medicine has seen so many. Two years in Egypt, however, brought him back to health again and he returned to Germany. He must have had a strong constitution for he is reported to have been a heavy drinker and smoker and to have lived generally a rather intemperate life. In spite of such habits and his contraction of tuberculosis, he lived to contribute much to science after his sojourn in Egypt. It was in 1890 that he returned to Germany and he was then associated with Koch at Berlin during the period of tremendously important bacteriological discoveries that were then under way. In 1896 he was made director of the Royal Prussian Institute for Serum Testing, a modest institution then, in spite of its important-sounding name. It was there

that he did his out-standing work, including his discovery of trypan red as a specific against trypanosomes. This work led eventually to the discovery of salvarsan and other synthetic drugs.

Ehrlich's great contribution to stain technology, however, was not in the latter part of his life but while he was still in his twenties; at that time, (1877–80) he was extremely interested in dyes and their staining action on blood and tissues. He was the first to realize the important difference between acid and basic dyes for staining purposes and at the same time showed that they could be combined with each other to form neutral stains. With one of the latter he stained blood more than ten years before Romanovsky devised the methylene-blue-eosin combination which led to the modern blood stains. By means of these neutral stains, he made very important contributions to the knowledge of blood cells, and his name would be known for this to-day, even if he had not done his more important work later in life.

WALTHER FLEMMING
1843–1905

WALTHER FLEMMING

Dr. Walther Flemming, who was for about 20 years Professor of Anatomy at the University of Kiel, was one of the founders of cytology and made many important contributions to cytological technic.

He was born at Schwerin in Mecklenburg, where his father, C. F. Fleming, who came of Flemish stock, was in charge of an insane asylum.

During the early part of Walther Flemming's life he was moving about so much that we rarely find him established at the same place two years in succession. He studied at Berlin and Rostock, and during the course of his student years suffered from a long siege of typhus fever. In 1869 he received a minor appointment at Würzburg in the field of zoology. In 1870 we find him at Amsterdam when the Franco-Prussian war broke out. During the war he saw service at Saarbrück and in 1871 returned to Rostock where he became a "Privatdozent" in the medical faculty. Altho he was at Rostock only a short time, he made important contributions while there on the structure of fatty tissue and other histological lines.

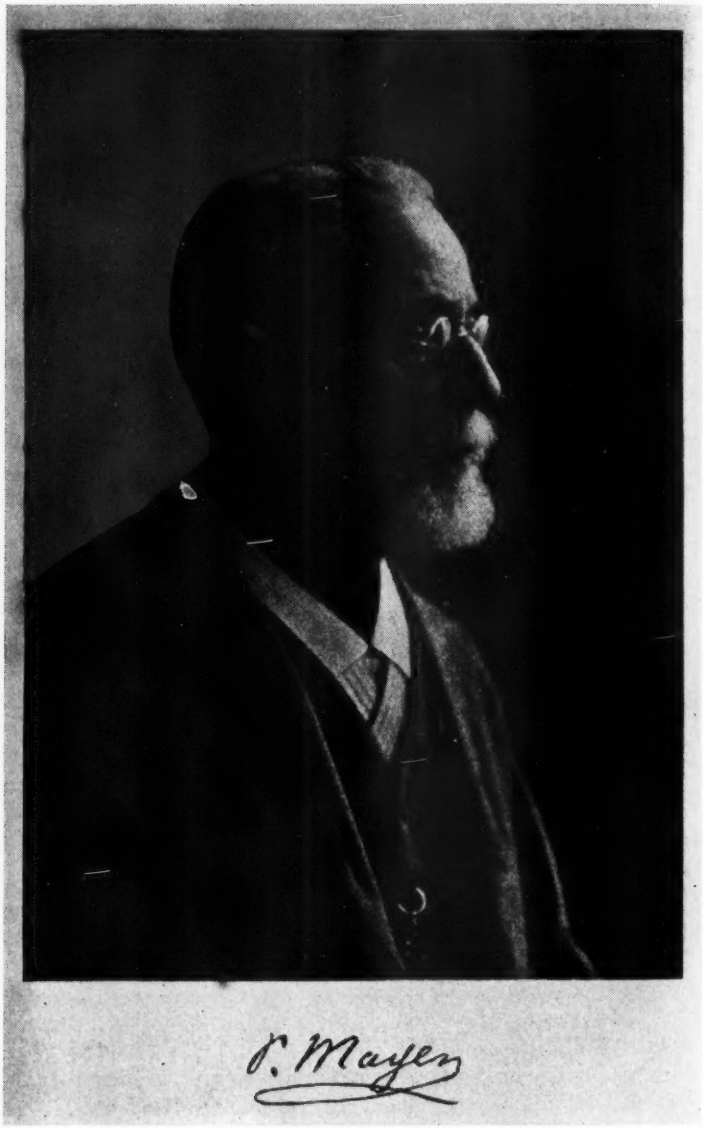
He left Rostock in 1872 to accept a more important position at Prague in the department of anatomy. He stayed there three years, the longest period he had yet spent at any one institution, and made further important histological contributions. He was not entirely happy during this period owing to hard feelings between the Germans and Czechs and was glad in 1875 to return to Germany. He went first to Tubingen, but we find him later that same year a professor at Königsberg.

In 1876 he accepted an appointment as Ordl. Prof. of Anatomy at Kiel and became director of the Anatomical Institute there, where he remained until his retirement in 1901. Now that he was finally located in a permanent position his work became even more outstanding and about 1877 he published his first papers on the cell and the function of the nucleus. It was at this time that he made his important contributions to cytology and to staining technic. In 1877, in fact, he studied the nucleus by overstaining and differentiating with alcohol by the method proposed by Hermann two years earlier. In 1882 he published his famous monograph on "Zellsubstanz, Kern und Kernteilung" and in 1884 his "Mitteilungen zur Färbetechnik." In 1891 he proposed the safranin-gentian-violet-

orange-G triple stain which has become so well known that it is usually understood to-day when anyone specifies the "Flemming stain."

His health began to fail in 1892 but he did not retire until 1901 and published papers up until 1902. He died August 4, 1905.

Dr. Flemming was characterized thruout his whole career by his keen mind and great energy. These characteristics are well illustrated by his striking productiveness during the early period of his career when he hardly remained long enough at any one institution to get settled. He was very independent, but unassuming and punctilious in the observance of his duties. He was always good-natured and even during the suffering of his later years is said to have been entirely uncomplaining. His contributions to cytology and histology were due not only to the methods he devised and the facts he discovered but also to his renown as a teacher and to the large number of young men who were thereby attracted to Keil to study histology.



PAUL MAYER
1848–1923

PAUL MAYER

There can be little question that one of the most important contributors to staining methods during the last decades of the nineteenth century and the beginning of the twentieth was Dr. Paul Mayer, who lived from 1848 to 1923 and spent the most active part of his life in the Zoological Station in Naples. Finding histological methods at the beginning of his career to consist of various quite unrelated procedures, he gathered them together and coördinated them so that later workers found a system of histological technic where none had existed before.

Dr. Mayer was born at Lüdenscheid and after attending schools at various places and serving as assistant apothecary in more than one location, he entered the University at Greifswald and took work in zoölogy and botany. His doctor's degree was taken at the University of Jena and immediately thereafter, 1877, he received his first appointment at the Zoölogical Station at Naples. This was only a temporary appointment followed by a brief stay at the Zoölogical Museum at Berlin; but the following year he received a permanent appointment at Naples where he stayed until 1913, 35 years later. From 1913 until his death he was located again at Jena.

When Dr. Mayer began his work in the 70's, microtechnic was in a state of transition. Instead of examining material unstained in water or physiological salt solutions, histologists were beginning at this period to mount preparations in glycerin, calcium chloride or Farant's solution. Chromic acid, Müller's solution and osmium tetroxide were coming to be used as fixing and coloring media, while some of the earlier staining formulae calling for carmin and hematoxylin were already known. Such procedures, however, were more or less individual undertakings on the part of their authors without any satisfactory systematization, nor were logical ways known of combining the different procedures. The greatest contribution of Paul Mayer was that he effected this systematization. There were, perhaps, other histologists of his period who had greater genius in devising new procedures, but none who contributed more toward putting the various histological methods in logical relationship to one another.

This does not mean that Dr. Mayer made no original contributions. On the contrary his work with carmin and hematoxylin has

given us many important staining solutions that are still in general use and bear the names which Dr. Mayer gave to them—such as, carmalum, hemalum, and hemacalcium. His contributions to the staining methods calling for these two dyes are, indeed, classic, and cannot be overlooked by anyone studying the history of staining. In spite of such contributions, however, one must undoubtedly agree with Péterfi[1] who, in reviewing Dr. Mayer's contributions shortly after his death, states that he must be regarded as the judge rather than the founder of microtechnical methods. Thanks to his critical judgment and his skill at improving the procedures of others, he combined all the methods which he tried into a single system of microtechnic which bore the stamp of his personality.

An outstanding contribution in this line was his service in connection with Lee's Vade-mecum. Whereas nearly any other scientist would have preferred getting out a laboratory manual bearing his own name, Mayer gave much of his energy to revising and enlarging a laboratory handbook originally written by someone else. This book was quite insignificant until Dr. Mayer began revising it, but after his work on it had begun, it became so useful that a new edition was called for every three or four years; and as each edition was greatly improved over the preceding, this manual soon became the authority that it still is to-day. His contributions published under his own name were too numerous to mention here. These publications appeared thru a period from the early 70's until 1920, only a few years before his death, and dealt with a considerable variety of zoōlogical subjects as well as with microtechnic. There is no question but that without his contributions the subject of stain technology would lack many principles and procedures which we regard to-day as fundamental.

[1]Péterfi, T. Paul Mayer. Ein Nachruf. 1948–1923. Zts. Wis. Mikr., **41**, 145–154. 1924.

NOTE ON A NEW METHOD OF DYE ANALYSIS

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ABSTRACT.—**Positive and negative dye ions may be titrated with one another conductometrically. The method appears to give values reproducible to about one percent. Difficulty has been experienced in preparing a satisfactory primary standard, so that data on the quantitative analysis of stains are not at present available.**

Since the time of Ehrlich (1898) and Romanovski (1891) it has been known that acidic and basic dyes, such as eosin and methylene blue, combine in some way to form a new compound, which may have properties quite different from either of the constituents (Spagnol, 1930). These "neutral stains" (Conn, 1929; see p. 142) have formed, since the time of Romanovski, the principal stains used in the clinical examination of blood, and have numerous other important applications. Since Holmes (1929) has shown that the combination between methylene blue and eosin takes place stoichiometrically, without appreciable adsorption of the dye present in excess upon the precipitate, it is surprising that the reaction typified by the formation of methylene-blue-eosinate has not been the subject of examination by conductometric methods. It is the purpose of the present paper to offer a preliminary report on such an investigation.

The subject is of particular interest inasmuch as it would seem to open up the possibility of a method supplementing the present rather cumbersome methods of dye analysis, of which titration with titanous chloride and gravimetric analysis by the precipitation of insoluble color acid (in the case of eosin dyes) are apparently in most general use at present. The titanous chloride method offers considerable difficulty because of the instability of the reducing solution, which must be kept under an atmosphere of hydrogen and used under an atmosphere of carbon dioxide. The gravimetric method is not of extremely wide application, being limited to dyes whose color acids or color bases may be quantitatively precipitated without the adsorption of extraneous ions by bringing the solution to the appropriate pH. It requires, moreover, several days for the completion of a series of analyses.

The procedure here outlined has the advantage of stability of solutions, rapidity of technic, and a fairly high degree of reproducibility. The dye solutions, kept in our laboratory for some months without special precautions except moderate protection from light and from bacteria and molds (with toluene in the case of acid dyes),

show little or no change in their titration curves. As for rapidity, an experienced technician can complete a titration and compute the end point within an hour without undue haste, or can obtain results of a fair degree of accuracy in 20 or 30 minutes.

The apparatus used includes an alternating current galvanometer and transformer capable of maintaining a potential of 6 or 9 volts with a frequency of 60 cycles from an ordinary 110 volt A. C. line, a resistance box with a capacity of one to 100,000 ohms in one ohm intervals, a slide-wire bridge, and a dip cell such as is frequently used in conductometric titrations. The usual Wheatstone bridge hook-up is used, the contact being set at the mid-point of the bridge so that the resistance of the solution between the electrodes of the cell may be read directly from the resistance box when the circuit is balanced.

In making the titration, the beaker containing the solution to be titrated and the dip cell is brought to a temperature a few tenths of a degree below the air temperature of the laboratory, and held at a constant temperature during the course of the titration. For rough work, the temperature range may be $\pm$ 0.05°C., but for the most precise work should not exceed $\pm$ 0.01°. Unfortunately the use of a temperature bath interferes with the titration, but with a little practice it is remarkably easy to hold the temperature to the desired degree of constancy, as evaporation from the surface tends to cool the solution slightly (hence, the initial temperature below that of the surrounding air) and the solution may be warmed slightly by laying the palm of the hand for a moment against the beaker. The initial resistance of the liquid in the cell is read, then titrating solution is added, usually in equal increments, from a pipette, with resistance readings after each increment. As a rule, the resistance may be read immediately after the addition of the titrating solution and subsequent stirring, but occasionally it may be necessary to wait for a few moments for the resistance to become constant.

The observed resistance is converted to conductance by taking the reciprocal, and may be further converted, if desired, to specific resistance by multiplying by the cell constant (0.773 for the cell used in securing the data given). This is unnecessary for the work in hand, however, because the constant difference introduced by neglecting the cell constant does not affect the calculated end point of the titration. The conductance is corrected for the dilution occasioned by the addition of the titrating solution by means of the usual dilution formula:

$$\Lambda_0 = \Lambda \times \frac{v}{v_0}$$

where Λ_0 is the conductance at the volume v_0, and Λ the conductance at volume v. The assumptions involved in the use of this formula are not serious, for each increment of volume is usually about 1/200 of the initial volume, and rarely greater than 1/50 so that the correction is not of large magnitude in any one case.

The corrected conductances are plotted against volume of titrating solution added, giving a function which generally comprises two straight lines connected by a curve. The straight lines, when produced, intersect at the end point. The data shown in Table 1 and plotted in the figure serve to illustrate this type of titration curve.

TABLE 1. SHOWING DATA FOR A TYPICAL TITRATION
5 cc. Pararosanilin with 1% Commercial Phloxine

Cc. Added	t	R	K (mhos)	K_0 (mhos)	$\triangle K$
0	24.80°	14,395	0.695×10^{-4}	0.695×10^{-4}	
1	24.80	13,196	0.759	0.762	$.067 \times 10^{-4}$
2	24.80	12,183	0.821	0.829	.067
3	24.80	11,282	0.887	0.899	.070
4	24.80	10,510	0.951	0.968	.069
5	24.80	9,861	1.015	1.037	.069
6	24.80	9,264	1.080	1.108	.071
7	24.80	8,715	1.149	1.183	.075
8	24.80	8,020	1.248	1.291	.108
9	24.80	7,370	1.358	1.410	.119
10	24.80	6,827	1.467	1.530	.120
11	24.80	6,347	1.577	1.651	.121

In the table, t represents the temperature in degrees C., K the measured conductance, K_0 the conductance corrected to the original volume, but not corrected for cell constant, and $\triangle K$ the change in conductance per unit of titrating solution added. The data given are for the titration of 5 cc. of pararosanilin acetate solution of unknown concentration with a 1% solution of commercial phloxine. The original volume of the solution was 230 cc.

As will be observed from Fig. 1, the end point of the titration lies between 7.0 and 7.2 cc. of phloxine added, depending on slight variations in the slope. The curve was intentionally selected from among the titrations showing the smallest angle of intersection between the lines (being thus most difficult to read precisely), and showing also the effect of some of the impurities encountered in commercial dyes which, in this case, simply increases the slope of both lines by the same amount. We can increase the accuracy of our result, however, and at the same time secure the end point more easily by referring to the equation of a straight line:

$$y = mx + C$$

where m is the slope (in this case simply $\triangle K$) and C the intercept

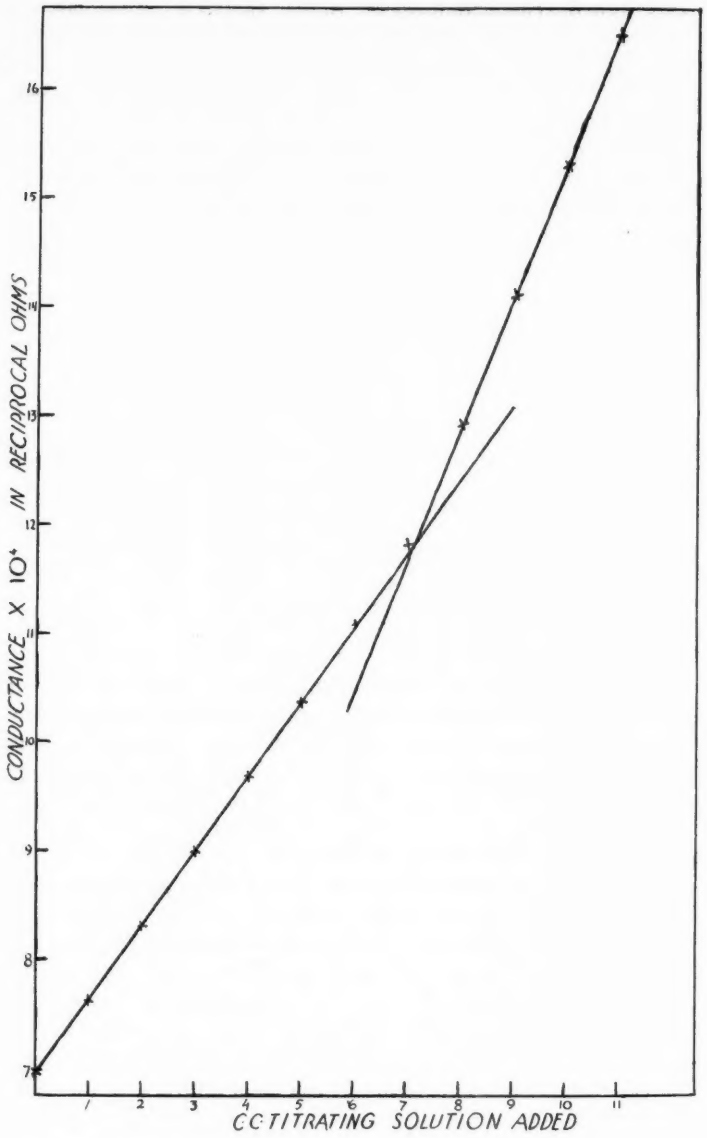

FIG. 1. Typical titration curve, from data of Table 1.

with the Y-axis. By applying this equation to the first few points having a constant $\triangle K$, and again to the last points having a constant $\triangle K$, and then solving the two equations simultaneously, we arrive directly at the value of x (i. e., cc. added) for the end point of the titration. For the data presented, this computation, taking $\triangle K$ for the first line as .069 x 10^{-4} and $\triangle K$ for the final line as .120 x 10^{-4} mhos, gives us an end point at 7.12 cc.

TABLE 2. REPRESENTATIVE RESULTS FROM TITRATING VARIOUS DYES WITH STANDARD PARAROSANILIN SOLUTION

Pararosanilin, C. I. No. 676 Erythrosin, C. I. No. 773
Phloxine, C. I. No. 778 Eosin, C. I. No. 768

Titration No.*	Soln. titrated	Titrating soln.	End point	Ratio Base: Acid
191	10cc. 778a	676acD	8.56cc.	0.856
193	10cc. 676acD	778a	11.79cc.	0.850
192	5cc. 773b	676acD	6.92cc.	1.382
194	10cc. 676acD	773b	7.26cc.	1.376
195	5cc. 768 liE	676acD	6.81cc.	1.361
196	5cc. 676acD	768 liE	3.65cc.	1.370

*The titrations are compared in pairs; in the first titration of each pair the standard pararosanilin is the titrating solution and in the second titration the unknown dye is the titrating solution; i. e., titrations are made in both directions.

TABLE 3. SHOWING THE EFFECT OF DYE ADSORPTION OR OCCLUSION BY THE PRECIPITATE ON TITRATION RESULTS

Erythrosine, C. I. No. 773 Crystal violet, C. I. No. 680

Titration No.	Soln. titrated	Titrating soln.	End point	No. of cc. 773 to 5cc. 680*
171	5cc. 773b (2%)	680acC Ia	5.27cc.	4.74cc.
177	10cc. 680acC Ia	773b	8.86cc.	4.43cc.
178	5cc. 680acC Ia	773c	8.83cc.	8.83cc.
179	10cc. 773c (1%)	680acC Ia	5.30cc.	9.43cc.

*It should be noted that the results of the second pair of titrations in this column should be divided by two to make them comparable with those of the first pair.

The method is not one of extreme precision, but furnishes values reproducible to about 1% as is seen from the data in Table 2. For convenience and brevity, the dyes are indicated by their Colour Index numbers, the letters serving to identify the particular solution.

The fact that the apparent relative concentrations of the reactants are the same regardless of whether anion or cation is being added serves to emphasize the results of Holmes already referred to (Holmes, 1929) in the matter of adsorption. If there were any adsorption of the dye ion present in excess on the precipitate, there would be a tendency toward too low a value for the end point, which is not ob-

served in this series of titrations. As a matter of fact, this tendency is observed in the titrations between crystal violet and erythrosin, as shown in Table 3. Other considerations make it appear probable that the adsorption here evidenced is of the same general nature as that observed in colloidal silver halides, and that the true values lie about midway between the two extremes.

The solution designated 773b is 2% erythrosin (89% dye content) and 773c is a 1% solution of a different sample of the same dye, secured at a different time, but bearing the same certification number from the Commission on Standardization of Biological Stains.

A serious difficulty has been encountered in the establishment of a primary standard. The problem may be attacked in several ways:

1. Titration against an inorganic ion whose concentration may be readily determined.
2. Quantitative analysis for nitrogen, bromine, or some other constituent present in constant amounts and readily determined.
3. Preparation of dyes in a very high degree of purity.
4. Titration with titanous chloride.
5. Gravimetric assay of insoluble color acid.

The first of these methods has been studied, but no ions have been found which combine stoichiometrically with the dyes without the formation of a precipitate which adsorbs quantities of ions present in the solution. The second has been used, but has been discarded because of the possibility of nitrogenous or other constituents as impurities, and because of the possibility of variations in halide content. The third has been attempted with some degree of success, but presents considerable technical difficulties, particularly in the acetates of the tri-phenyl methane dyes, and in the lithium salts of the eosins. The time and materials required for the fourth method are not available at present, altho the method seems to offer the most promising mode of attack. The fifth has yielded some information, and is being employed wherever it is applicable. The problem still awaits satisfactory solution.

As has already been noted, the impurities normally present in commercial stains do not appear to affect the accuracy of the titration except as they increase the total conductance and make the actual resistance change due to combination between dye ions smaller, i. e., the end point is not affected but the angle between the two lines is flattened out making the reading more difficult. The reaction does not seem to exert any marked effect as long as the titrations are conducted between about pH 5 and pH 7, the usual pH-range of aqueous solutions of the commercial stains employed. It is, of course,

obvious that the solutions used must both have pH-values on the same side of neutrality.

In conclusion, it may be stated that the method, which seems to include tri-phenyl methane and xanthene dyes, together with some, at least, of the thiazins and sulfonated dyestuffs, has not as yet been satisfactorily applied to the quantitative analysis of dyes, largely because of difficulties in preparing a primary standard. In view of the precision with which an individual titration may be reproduced, however, the technic seems to merit further investigation.

BIBLIOGRAPHY

CONN, H. J. 1929. Biological Stains. 2nd Ed. Publ. by Commission on Standardization of Biological Stains, Geneva, N. Y. p. 142.

EHRLICH, P., and LAZARUS, A. 1898. I Abt. In Nothnagel's Spec. Path. Ther., Bd. **8**, Vienna (after Conn, 1929).

HOLMES, W. C. 1929. The chemical analysis of thiazin eosinates. Stain Techn., **4**, 49.

ROMANOVSKI, D. L. 1891. On the question of parasitology and therapy of malaria. Imp. Med. Military Acad., Dissert. No. 38, St. Petersburg (after Conn, 1929).

SPAGNOL, G. 1930. Comportamento farmacologico dei complessi di sostanze coloranti vitale. Arch. d. Sci. Biol. (Italy), **15**, 216.

APPARATUS FOR THE SIMULTANEOUS HANDLING OF NUMEROUS HISTOLOGICAL SPECIMENS

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ABSTRACT.—**An apparatus has been devised consisting of a series of bakelite plates $2\frac{3}{4}$ inches in diameter which can be clamped together one on top of the other. Each contains 37 depressions $\frac{1}{4}$ inch in diameter, with a $\frac{1}{16}$ inch hole at the bottom of each passing completely thru the plate. Each depression can contain its own specimen, its identity established by the number of the plate and the position of the depression. Several plates can be clamped together and run simultaneously thru the necessary reagents, thus handling large numbers of specimens without losing the identity of any of them.**

Considerable horticultural research has recently been devoted to the study of flower bud initiation and development. In order to study microscopically the progressive development of the buds on a growing shoot it is necessary to collect and keep the buds separate and in serial order.

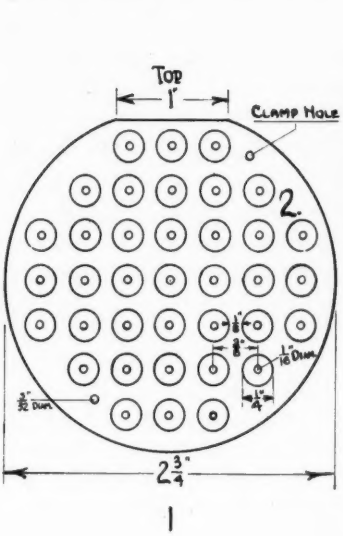
Heretofore, the identity of the buds has been maintained by placing each in an individual vial at the time of collection and by transferring it to successive vials during subsequent processes. If the buds require treatment with hydrofluoric acid it is necessary to use vessels impervious to this substance. Glass vials, coated with paraffin, are frequently used. The task of maintaining the identity of the buds requires considerable time if one is to study a large quantity of buds.

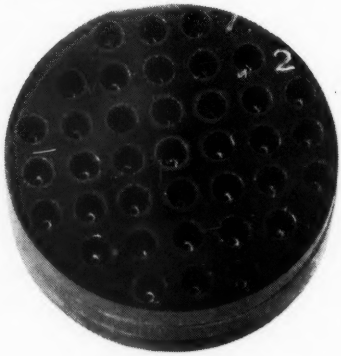
While working out a project involving a study of approximately 2000 buds, the author developed an apparatus to minimize the routine work. This apparatus is made from $\frac{1}{4}''$ sheet bakelite and No. 16 nichrome wire, bakelite and nichrome having been found by tests to be resistant to the chemicals used in preparing the buds for sectioning.

The apparatus is constructed by cutting the bakelite into circular plates $2\frac{3}{4}''$ in diameter, and by making individual compartments in these plates for the buds. The arrangement of the compartments shown in Fig. 1 permits a maximum number (37) per plate. Construct them by drilling $\frac{1}{4}''$ holes three-fourths of the distance ($\frac{3}{16}''$) thru the plates, and then by drilling $\frac{1}{16}''$ holes entirely thru the plate in the centers of the bottoms of the $\frac{1}{4}''$ holes. The small holes permit the passage of reagents from one compartment to another. Any desired number of these plates may be fastened together and manipu-

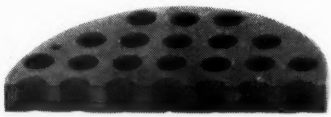
lated as a unit. For this purpose drill two additional holes (clamp holes) thru the plate in the positions indicated (Fig. 1).

The numbering of the compartments is obviated by constructing a secant 1″ long, parallel with the rows of compartment holes. Consider the secant as the top of the plate (Fig. 1) and without putting any numbers on the plate, number the compartments in the same order as reading matter appears on the printed page. Complete the plate by stamping a number on the top surface (Fig. 1).





2



3

Fig. 1. Diagram showing arrangement of depressions in bakelite plate.

Fig. 2. Photograph of an entire plate.

Fig. 3. Photograph of plate cut in two along median line to show depth of depressions.

When large quantities of buds are being studied it is necessary to use several plates. These may be stacked together and held in place by running the wire thru the clamp holes and bending it as indicated (Fig. 2). With an empty plate as a cover to the top plate containing specimens, this series of plates may be placed in a 400 cc. beaker where the various changes of reagents are made. In removing the cover from the tier of plates, and each successive plate after the buds have been carried thru the complete series of reagents preparatory to casting, the investigator must take care to slide the cover off rather

than lift it vertically. Otherwise the buds will adhere to its lower surface and their identity will be lost.

This apparatus offers several advantages to investigators studying the chronological and serial development of large quantities of buds. The small cell compartments and the size of the plates, as they fit into a 400 cc. beaker, afford considerable saving of reagents. Another saving, and perhaps the one of major importance, concerns the work in preparing the buds for sectioning. By using the simple apparatus just described the investigator may reduce his routine work at least one-half and may thus devote more time to the real objectives of his research.

PREPARATION OF PALAEOBOTANICAL SECTIONS BY THE PEEL METHOD

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ABSTRACT.—**The use of nitrocellulose peels in the study of petrified plant material led to experimentation with various nitrocellulose solvents used commercially in the manufacture of lacquers. The following solution of nitrocellulose gave the most satisfactory results, the film produced being smooth, free from bubbles, and little affected by moisture: 20 second nitrocellulose, 20 g.; butyl acetate (Tech.), 200 cc.; tricresyl phosphate or methyl phthalate, about 1 cc.; toluene or xylol 10 to 20 cc.**

For the mechanical procedure in the making of sections by means of nitrocellulose peels, reference is made to Chamberlain "Methods in Plant Histology" Fifth Revised Edition, 1932.

The remainder of the paper deals with the effects on the resulting film of the various grades of nitrocellulose, the various solvents used, and plasticisers and other substances which may be added to the solution.

The cellulose peel method of preparing palaeobotanical sections was devised by Walton and Koopmans (1928). It has been used, with slight modifications, by Hemingway, Gordon, and others. The method, in brief, is as follows: A plane surface of the petrifaction is etched with an acid. This removes a thin layer of the matrix, leaving the carbonaceous material standing in relief. A solution of a cellulose compound is poured over the surface. After the evaporation of the solvent, the film, with the embedded carbonaceous material may be peeled off and used as a thin section.

With the following exceptions, the schedule as given by Krick (1932) or by Chamberlain (1932) was followed:

1. A different solvent was used for the cellulose.
2. As the solvent used gave a film which adhered to the rock surface much more closely, the etching time for the same material was considerably shorter. The acid used was 5 parts of commercial hydrochloric in 95 parts of water. Time of etching was 30 to 60 seconds.
3. It was not necessary to moisten the surface of the block with the solvent prior to pouring on the cellulose.
4. The film was left on the block until thoroly dry.

The dry film is removed from the block by lifting the edges away from the block with the corner of a safety razor blade and then gently

peeling it off by means of the fingers. It should be washed for a minute or two in dilute hydrochloric acid to remove any adhering mineral, and then in water to remove the acid. It should then be placed between blotting paper or newspaper in a letter press for about 24 hours in order to insure a plane surface. It may be used, either mounted or unmounted, as a thin section. Most sections show the detail much better when mounted in Canada balsam between slide and cover glass. The writer found Eycleshymer's clearing fluid (equal parts of bergamot oil, cedar oil, and carbolic acid) preferable to xylol as a clearing agent preparatory to mounting in balsam, since there is less tendency for the film to warp.

By far the most important factor in making successful films is the solvent, or mixture of solvents used. It is on this point that the writer wishes to make a few observations.

A year ago, when the writer began his studies of coal ball material, the method used in the laboratory was that described by Krick and by Chamberlain. Celloidin, or a 4 to 6% solution of parlodion in ether-alcohol, was the substance used for films. This solution is subject to the following disadvantages:

1. It is difficult to get a film without a number of small bubbles.
2. The film must be carefully watched, removed from the block before it is quite dry, and placed immediately between glass plates or between blotters in a press in order to prevent the film from wrinkling. Even then it frequently becomes wrinkled at the edges. If the film is left on the block to dry it wrinkles away and curls and shrivels so badly that it is ruined. This tendency to wrinkle away from the block may be entirely overcome by the use of a "plasticiser" but the author was not acquainted with this at that time.
3. As the film does not adhere to the surface of the block as closely as in the method to be described, it is necessary to etch much deeper. This permits a greater chance of losing part of the freed carbonaceous structure in the acid and wash water.

Solutions of cellulose acetate in acetone and of transparent celluloid in acetone or amyl acetate or mixtures of the two, as suggested by Hemingway and Gordon, were then tried out. The celluloid in amyl acetate made good films, but the other solutions were failures. Bubbles were formed in the films from mixtures of acetone and amyl acetate. Acetone films usually had bubbles and invariably formed ridges, due to too rapid evaporation and to surface tension effects. Also, they often became milky due to absorption by the acetone of moisture from the air.

Inquiries in regard to nitrocellulose, solvents, etc., as used commercially in the manufacture of fast-drying lacquers, led to an acquaintance with plasticisers. A plasticiser is a non-volatile liquid added in small quantity to the solution in order to make the resulting film more pliable. Upon evaporation of the solvent, the plasticiser remains in the film. Without plasticisers, nitrocellulose films become hard and somewhat brittle with age. Too much plasticiser in the film, however, makes it adhere too closely to the block, with consequent danger of tearing the film in the process of removing it. The following are some of the commercial plasticisers: Dimethyl phthalate, dibutyl phthalate, castor oil, tricresyl phosphate (Lindol), and triphenyl phosphate. Of these, only dimethyl phthalate and castor oil have been tried on the films. The phthalate was preferable to the castor oil. It is claimed that the phosphate plasticisers give films of higher tensile strength than the other plasticisers.

Butyl acetate (tech.) is to be preferred to amyl acetate as a solvent as it is faster drying. The vapor is also less irritating to the tissues of the nose and throat. A little xylol or toluene added to the solution appears to aid in making the film easier to peel off.

Celluloid itself is rather unsatisfactory as a source of nitrocellulose, as the film becomes yellow within a few weeks. Pure nitrocellulose, which may be obtained from any powder company, is free from this fault. The nitrocellulose comes in cans, looks like cotton, and is moistened with denatured alcohol. This material was used without previously allowing it to dry. The following formula was used:

20-second nitrocellulose	20 g.
Butyl acetate (tech.)	200 cc.
Methyl phthalate	about 1 cc.
Toluene or xylol	10 to 20 cc.

This solution has about the consistency of glycerin. The films take from 4 to 6 hours to dry. If one is not in a great hurry for the sections, it is better to allow the film to remain on the block for 1 or 2 days to permit the cellulose to set completely.

By the use of nitrocelluloses of low viscosity, such as ½-second or ¼-second nitrocellulose, good films may be obtained with solvents of a higher evaporation rate. The low viscosity helps prevent the formation of ridges and bubbles. The solutions used are much more concentrated, but even so, they run more freely. The following schedule gave good results:

½-second nitrocellulose	40 g.
Solvent	200 cc.
Plasticiser	2 cc.
Toluene	20 cc.

The solvent used consisted of 1 part of butyl acetate to 2 parts of either acetone or ethyl acetate (tech.). These films dry in 2 to 3 hours. They are somewhat inferior to those made from 20-second stock, as they have a lower tensile strength and are thus more subject to tearing during the stripping process.

REFERENCES

CHAMBERLAIN, C. J. 1932. Methods in plant histology. 5th revised ed. 157–158.

KOOPMANS, R. G. 1929. Celluloidpreparat anstatt Dünnschliff. Jaarverslag over 1928. Geol. Bur. Netherlands, Heerlen. pp. 131–132.

KRICK, HENRIETTE VALLETTA. 1932. Structure of seed-like fructifications found in coal balls from Harrisburg, Illinois. Bot. Gaz., **93**, 153–154.

LECLERCQ, SUZANNE. 1928. La Méthode J. Walton pour la Préparation des Lames Minces. Annales de la Soc. Géol. Belg. t. **52**.

NOE, A. C. 1930. Celluloid films from coal balls. Bot. Gaz., **89**, 318–319.

WALTON, J., and KOOPMANS, R. G. 1928. Preparation of cellulose films and their use in making serial sections of coal ball plants. Rept. British Assn. at Glasgow. pp. **615** and **688**.

WALTON, J. 1928. A method of preparing fossil plants. Nature, **122**, 571.

COLORIMETRIC TESTS OF NUCLEAR STAINING[1]

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ABSTRACT.—**The materials of these investigations were the mucosal nuclei of formalin-paraffin sections (stained by hematoxylin and eosin) from the human uterus in carcinoma, non-malignant hyperplasia and in health. By a scale made of water color pigments placed near the magnified images of nuclei projected on a screen, it was demonstrated mathematically that, for a given specimen, nuclear staining intensity varies inversely with nuclear volume. The most probable explanation is the greater separation of chromatic particles in the larger nuclei.**

Colorimetric study of ordinary histological material has not been developed to any great extent, and yet crude quantitative judgments are frequently heard, wherein it is stated that one specimen or disease shows more darkly stained nuclei than another. It is obvious that in such comparisons the value of the results must depend on the uniformity of the staining technic and of the thickness of the sections. This is doubtless one reason why colorimetric tests are not in vogue; another reason being the difficulty of comparing a stained specimen, viewed thru a microscope, with a color-scale exposed to the same intensity of illumination. This second difficulty can now be overcome by use of the projection method of examining specimens, which is employed when large numbers of nuclear measurements are required. Under these conditions some colorimetric tests were recently carried out as part of a quantitative study of epithelial nuclei in hyperplasia and carcinoma of the mucosa of the body of the human uterus. A preliminary note of these investigations appeared in the Proceedings of the American Association of Anatomists[2] and a lengthier report is now in press.[3] The subject has, however, a much wider application than is exemplified in these reports, and further investigations have revealed more conclusive results than were therein set forth. The purpose of the present paper is to indicate the nature of these results.

[1]The work recorded here is part of a series of investigations rendered possible by a generous grant from the Banting Research Foundation, Toronto, Canada.

[2]Mainland, Donald. Nuclear sizes in the endothelium of the body of the human uterus in malignant and non-malignant disease and in health. Anat. Rec., **52**, No. 1, Suppl. 67. 1932.

[3]Mainland, Donald. A quantitative study of epithelial nuclei of the body of the uterus in carcinoma and in non-malignant hyperplasia. Trans. Nova Scotian Inst. Sci., Halifax, Canada (in press) 1933.

A summary of the previous work may be given as follows: The objects of the investigations were: (a) to show whether differences in disease were reflected in nuclear staining intensity in tissues prepared by routine pathological methods for diagnosis; (b) to show the relationship in individual specimens between nuclear size and staining intensity. Formalin-fixed paraffin sections stained by hematoxylin and eosin were obtained from fifty specimens of human uterine mucosa, in carcinoma, in hyperplasia and in the healthy state. Nuclear images were projected (X 1520), traced and measured. Nuclear thickness was determined by the fine adjustment of the microscope. A series of five purple water color paints (lettered A, B, C, D and E) on cardboard formed the scale whereby the staining intensity was classified, the card being held near to each nuclear image in turn. Numerical equivalents for the letters were obtained[4] by comparison with water color mixtures of known dilution, and biometrical correlation tests were applied. The results showed that, in any one specimen, staining intensity tends to vary (a) directly with nuclear thickness, and (b) inversely with nuclear breadth. It was further shown that, as pathological technic is now carried out, differences of nuclear staining intensity cannot be used for fine differentiation between specimens; but, if fixation, sectioning and staining were more standardized, the tests would very probably be of value in this respect, for there were significant size differences between carcinoma nuclei and the nuclei in non-malignant hyperplasia, and, as stated above, size and staining intensity are correlated in individual specimens, i. e., where the staining technic is uniform.

Of the two correlations, (a) and (b), just referred to, the former was easily explained, because a thicker nucleus would naturally tend to stop the light rays more than a thin one and, therefore, would appear darker. The negative correlation between breadth and staining intensity was not, however, so easily explained. It might reasonably be asked, whether the connection between breadth and staining intensity were not merely another expression of the relationship between thickness and staining. A nucleus might be broad because its long axes (breadth and length) were in the plane of the section, while at right angles it might be thin. Such a nucleus would stain palely, and this would account for the negative correlation between breadth and staining. If this were the explanation, however, it should be possible to demonstrate a significant negative correlation

[4]The author wishes to express his thanks to Professor H. L. Bronson of the Physics Department, Dalhousie University, for his interest in this part of the work and for his various suggestions regarding the methods of standardizing the colorimetric scale.

between breadth and thickness, and it was shown that such a correlation did not exist. The connection between breadth and staining must, therefore, be independent of the connection between thickness and staining.

It had been shown that breadth and nuclear volume (calculated by the formula for an ellipsoid) were positively correlated, i. e., in general, greater breadth represented greater volume; and it was suggested, as a conclusion of the investigations referred to (see footnote 3), that the greater the nuclear volume, the greater would be the spreading apart of the chromatic particles and the paler would be the staining. Further steps have now been taken to substantiate this suggestion, by ascertaining more directly the relationship of volume and staining.

In the first place, altho the volumes were determined for only five specimens, the results appeared to agree in the main with the suggestion just stated. The situation was complicated, however, for certain nuclei were large because they had great thickness and, therefore, would stain deeply, whereas other nuclei were large because of great breadth, in virtue of which they would stain palely. The direct application of a correlation test could not, therefore, show clearly the essential connection or lack of connection between volume and staining. The influence of thickness on staining had to be neutralized, and this was done by dividing the staining intensity (expressed numerically as stated above) by the thickness. Thus if a nucleus had a thickness of 5μ and a staining intensity of C (i. e., 2.5 units) the result would be 0.5. This would represent, as it were, the staining intensity per unit thickness. The correlation between volume and the staining intensity (thus corrected for thickness) was very definite (-0.49; standard error: $\pm$ 0.054), *i. e.*, as the volume increased, the staining intensity tended to decrease. There has been obtained therefore, a direct mathematical relationship between nuclear volume and staining intensity.

Discussion

It may, perhaps, be remarked that the work recorded here was only a very laborious method of arriving at a result that every histologist already knew, in a general way,—the tendency for large nuclei in a section to stain more palely than smaller nuclei. The new fact—that this relationship is open to mathematical demonstration—suggests, however, that it may be used to help in the investigation of nuclear structure (both in the fixed and in the unfixed state), for such investigation must become more and more mathematical.

The most reasonable explanation of the relationship established is that the larger the nucleus, the greater the spreading apart of the stained particles and, consequently, the paler the total staining intensity. This explanation further suggests that the chromatic material in the various nuclei is the same in amount in any particular specimen. Before this suggestion can be accepted, however, it must first be determined why the correlation is only about -0.5, instead of a perfect negative correlation (-1.0), which it should be if size were the only factor influencing staining intensity. To a considerable extent, doubtless, the discrepancy is due to lack of precision in the method of color estimation, and to the influence of variations in density of the medium in which the specimens were mounted, the presence of thicker or thinner parts of the tissue near the nucleus under observation, and so on.

NOTES ON TECHNIC

CONCERNING FAST GREEN.—Fast green dissolved in either 95% alcohol or in clove oil offers a very satisfactory counterstain for hematoxylin, for Feulgen's reaction, or for differentiating safranin (Haynes). It is a fairly strong stain and 0.25% solutions seem to stain sufficiently deeply.

Fast green can also be used for the differentiation of lignin, as erythrosin is used in Jackson's technic,[1] following crystal violet. Also for the differentiation of chromatin in sections stained with crystal violet. Tested for the two latter purposes, good results were obtained with plant material following Flemming's, Feulgen's. Gilson's and the solution of Ruffini.

As a counterstain for hematoxylin and Feulgen's reaction on both plant and animal material it gives good results on most commonly used fixatives. Dissolved in alcohol it is a satisfactory stain for erythrocytes or for blood smears treated by Feulgen's method.—LUBOV A. MARGOLENA, Geneva, N. Y.

NOTES ON THE STAINING OF TUBERCLE BACILLI IN SPUTUM.—Doglio's method, calling for brilliant yellow as a counterstain against acid-fast organisms, (abstracted on p. 76 of the present issue of this journal) has been tried out in our laboratory with various batches of fuchsin. The brilliant yellow used was that of the National Aniline & Chemical Co., Colour Index No. 144.

The method can be well recommended for wide application in pathological laboratories because of its quickness, ease, and particularly because of the boldness with which the organisms stand out on the lemon background. The possibility of questioning whether an organism is acid-fast or not, when the bacteria in question lie on, under, or between leucocytes, is entirely eliminated by this technic. Altho excellent for diagnostic purposes, the method does not, however, give as good a picture as the older procedure, by which a clear presentation of the whole flora is obtained, and the leucocytes are definitely outlined against mucous or debris.

In this connection it may be well to add that alkaline methylene blue is much improved by using in greater dilution than the standard formulae. Plain aqueous or aqueous alcoholic methylene blue has proved to be at least as good as the alkaline solution. In fact, the red bacteria sometimes seem clearer against the natural blue-green background of methylene blue than against the bluish-purple produced by the alkaline solution.—LUBOV A. MARGOLENA, Geneva, N. Y.

[1]Jackson, Gemma. Crystal violet and erythrosin in plant anatomy. STAIN TECHN., 1, 33. 1926.

Stains Recently Certified

In the table below is given a list of the batches of stain approved since the last one listed in the January number of this journal.

Stains Certified December 1, 1932, to February 28, 1933*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Congo red.....	CQ 2	69%	As histological counterstain	Dec. 3, 1932
Sudan III.....	LY 2	66%	As fat stain	Dec. 19, 1932
Anilin blue W. S.	LK 1	....	For general histological staining	Dec. 23, 1932
Light green SF yellowish.....	NL 6	85%	For general histological staining	Jan. 16, 1933
Eosin Y.....	LE 8	75%	For general histological staining and as constituent of blood stains	Jan. 19, 1933
Wright's stain...	LWr 3	....	As blood stain	Jan. 20, 1933
Methylene blue.	LA 4	80%	As bacteriological and histological stain and as constituent of blood stains	Jan. 21, 1933
Crystal violet...	LC 6	88%	As histological, cytological and bacteriological stain	Jan. 26, 1933
Eosin Y.....	NE 9	92%	For general histological staining and as constituent of blood stains	Feb. 8, 1933

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

LABORATORY HINTS FROM THE LITERATURE

Under this heading are given interesting new methods in microscopy that have been noticed in other publications. The readers' assistance is urged in calling the editor's attention to any outstanding methods in this field that are overlooked. Thru coöperation with the Wistar Institute of Anatomy and Biology of Philadelphia, these same abstracts are to appear on the Laboratory Service Cards of that institute. Those desiring some of these cards without subscribing for the entire Bibliographic Service of the Wistar Institute, can order from STAIN TECHNOLOGY, at a price of 6 cents each.

BAKER, J. R. A new method for mitochondria. Nature, **30**, 134. 1932.

Quinone is recommended for the demonstration of mitochondria. The method has been worked out mostly with the liver of the newt and with the kidney of guinea pigs.

Treat tissues for an hour with parabenzoquinone dissolved in saline solution, from 0.05 to 0.5%, depending on the delicacy of the material. Use a saline solution roughly isotonic with the blood of the animal; but if the quinone is used at 0.5%, make up the saline 0.1% weaker. Fix in Carnoy's fluid without the chloroform, or in Bouin's. Stain with iron hematoxylin or by Altmann's or Benda's method. For Altmann's staining, a convenient differentiator is 1 part of absolute alcohol saturated with picric acid to 7 parts of 30% alcohol. No special mordanting is required for subsequent staining in Benda's alizarin and crystal violet, which gives good results after Carnoy's fluid. Other quinones offered no advantages over parabenzoquinone.

CHURCHMAN, J. W. and EMELIANOFF, N. V. A new method for staining bacterial capsules. Proc. Soc. Exp. Biol. & Med., **29**, 514–515. 1932.

By means of this simple method capsules may be demonstrated about bacteria. Flood air-dried smears with 10 drops of Wright's stain. Leave until it has nearly, but not completely, evaporated to dryness when a pinkish color replaces the original blue. (this usually takes about 3 to 4 minutes). Wash off as rapidly as possible with Clark's and Lub's buffer solution (pH 6.4 or 6.5); distilled water is sometimes preferred. Dry with fan without blotting. If washing is done before the stain has evaporated the capsule-like structure will not be stained. If desired, stain overnight, then remove and allow stain to evaporate, dip in buffer and dry without blotting.

Several stains of *Diplococcus pnemoniae*, *B. anthracis* Koch, *Klebsiella pneumoniae* Friedlander showed pink capsules against the blue organisms. The structure may apparently be made to disappear from *B. anthracis* by suspending the organisms in 1% NaCl and then staining. It reappears, however, after the bacteria are washed free from the salt and restained. Precipitation of the stain may cause granular deposits and ring-like structures, but careful study reveals these as artefacts.

CHURCHMAN, J. W. and EMELIANOFF, N. V. A study of the bacterial capsule by new methods. J. Exp. Med., **57**, 485–510. 1933.

The technic employed in this work is essentially the same as in the paper cited just above. In addition to the method given in the preceding paper, certain modifications of Wright stain are tried: the substitution of erythrosin in certain cases has proved satisfactory, altho the preparations are not permanent; the substitution of ethyl alcohol or glycerin for methyl alcohol as a solvent gives excellent results; the use of the tetrachrome stain or Giemsa's stain sometimes gives good results. Some success has also been obtained with Casares-Gil's flagella stain.

The paper discusses the findings at some length, with photographs and drawings to illustrate the points brought out. The method produces appearances resembling capsules around the somata of a number of non-capsulated organisms.

CONN, H. J. The Cholodny technic for the microscopic study of the soil microflora. Zentbl. Bakt., **87**, 234–239. 1933.

The direct microscopic method proposed by Cholodny in 1930 shows very plainly the characteristic colony formation of soil organisms and brings out so strikingly the occurrence of some types in association with others, that the wide use of the method may disclose various secrets of soil microbiology. By means of this method one may

observe the change in the predominating flora of a soil from fungi or Actinomyces to bacteria or vice versa.

The procedure here adapted is that of Cholodny with only minor modifications. Instead of digging a trench in the field soil for inserting the slide, 2 slides are placed in covered jelly tumblers filled with soil. Cholodny recommends 2 weeks incubation, but the writer has found most interesting results after 5 to 7 days. Usually one slide is removed in about 5 days, and a new one is put in its place. The second slide is removed in about 7 days after insertion, and the slide inserted where the first was removed is taken out after being in place 5 or 6 days.

Cholodny's erythrosin has been replaced by rose bengal in this work, (rose bengal, 1 g.; calcium chloride, 0.01 g.; 5% aqueous phenol, 100 cc.). The slides are laid preferably on a flat surface over a boiling water bath, the stain poured upon them, and allowed to stay 1 minute, taking care not to let them become dry; or, they may be stained by immersing in jars of steaming stain solution.

CUYLER, W. K. A differential stain for dried, unfixed vaginal smears. J. Lab. & Clin. Med., **18**, 314-315. 1932.

The following stain is recommended for studies of the complete estrous cycle of the white rat. Five vaginal smears, in ½ inch areas, are made at different times on a single slide and allowed to dry. The slides are immersed in 95% alcohol for 3 minutes, then in distilled water for 1 minute, then in Harris' or Delafield's hematoxylin for 20 minutes; they are then washed in tap water for 2 minutes, in alkaline alcohol (70% alcohol made by using a 4% solution of aqua ammonia instead of distilled water) and counterstained 8 hours or over night in the following: Indigo-carmin (Grübler) 0.25 g., eosin Y (Coleman and Bell) 1 g., distilled water 99 cc., with a pinch of thymol added. The slides are washed quickly in tap water, dehydrated in alcohol, cleaned in xylol and mounted in balsam.

Results: Nuclei of epithelial cells gray, cytoplasm lavender; cornified cells bright pink; nuclei of leucocytes dark, cytoplasm clear; red blood cells pink.

DEFLANDRE, G. Sur l'introduction de la nigrosine dans la technique des frottis secs d'Infusoires. Ann. de Protist., **2**, 121–124. 1929.

The writer calls attention to several recent papers in which methods are reported for the negative staining of infusoria in dry smears. He states further that he himself described in Bul. Soc. Botan. de France, **70**, 738–741, 1923, a technic in which nigrosin was used for the study of lower algae. Reference to the earlier paper shows the technic to be as follows: Place a drop of the organisms, either living or in the fixative, on the slide and add a drop of aqueous nigrosin (5 to 10%). Mix and spread uniformly. Dry as fast as possible—in a draft, or by fanning the slide. Mount in Canada balsam.

By means of this quick method permanent smears of the organisms showing external morphology are obtained. Photomicrographs of these preparations surpass those prepared by other methods.

In the present article the addition of a little acid fuchsin to the nigrosin solution is recommended. This colors the organisms pink in a gray background. If mere traces of acid fuchsin are added the infusoria remain colorless for a few days, after which they assume a pink coloration on a gray background. If sufficient fuchsin is added to stain the protozoa instantly, the color will become so intense in a short time that the preparations will be unusable for several years. After such a period they will fade and show excellent staining. Since the fuchsin will continue to fade in the balsam, however, the preparations do not seem to have permanent value.

The writer has also employed Lyon's blue (aniline blue, water soluble) but does not recommend it. He also tried phloxine and rhodamine which are claimed by Breslau to stain the nuclei, but he finds them less satisfactory than acid fuchsin.

DOGLIO, P. Rapid staining of tubercle bacilli in sputum. Giornale di Batteriologia e Immunologia, **8**, 243. 1932. (The present abstract is made from a note in the J. Am. Med. Assn., **99**, No. 6, 514, 1932.)

Steam the smear with the usual Ziehl-Neelson's carbol-fuchsin. Wash thoroly in running water. Counterstain and differentiate 40 to 50 seconds with the following solution: Brilliant yellow 0.15 g., concentrated sulfuric acid 10 cc., alcohol 20 cc., water 85 cc. Wash thoroly and blot.

The bacilli are red on a lemon-yellow background. The method is an improvement over the Ziehl-Neelson technic, as it is more rapid, and reveals the organisms more clearly, thus facilitating diagnosis. It is just as reliable as the usual procedure.

DOUGLAS, M. G. Cooper's modification of the Ziehl-Neelson staining method as applied to tubercle bacilli in tissue. J. Lab. & Clin. Med., **17**, 1131–1132. 1932.

The method is an application of the Ziehl-Neelson staining for the tubercle organism in paraffin sections. Place slides in an incubator at 37°C. for 2 hours, or overnight in Ziehl-Neelson carbol-fuchsin (formula not given) with 3 cc. of a 10% aqueous Na Cl per 100 cc. of the solution. Transfer to ice-box for 30 minutes. This will precipitate the stain. Wash in water and decolorize for about 1 minute with 5% nitric acid in 95% alcohol.

Wash and stain with methylene-blue-azure for 10 minutes. For this stain, prepare 1% azure II, and Mallory's 1% methylene blue in 1% borax; for use, mix in equal parts, and dilute with 18 parts of water. This diluted stain should be used freshly prepared. After staining, wash in water, differentiate in 95% alcohol with colophonium, dehydrate, clear and mount.

The author found that by means of this method 1.4 times more bacilli are counted than by the usual procedure. He does not, however, give the source or exact chemical nature of the dyes used.

FINK, H. and WEINFURTNER, F. Die Methylenblaufarbung von Hefezellen und ihre Beziehung zur Wasserstoffzahl und zu dem Permmeabilitatsproblem. Wochenschr. Brauerei, No. 9, 10, 11, 1930. (Abstracted from Zts. Wis. Mikr., **49**, 268–270. 1932).

The authors show that the use of 1:10000 solution of methylene blue is not reliable for differentiation between live and dead yeast cells. Under certain conditions the dead cells will stain, or vice versa.

Lebedeff's dry yeasts, known for high content of dead cells, were placed in a 1:10000 solution of methylene blue of various reactions—pH 2, 4, 6 and 8. Less than 1% were stained at pH 2, rather more at pH 4 or 6 and almost all at pH 8. The intense staining at pH 8 is regarded as adsorption of the poorly soluble stain base, which developed in the alkaline medium from the methlyene blue hydrochloride. Besides the reaction, the dispersion of the stain may influence the results, and also the formation of salt between the methylene blue base and the acid proteins of the yeast.

The staining intensity varies according to whether distilled water or tap water is employed. If distilled water is used for 30 minutes and methylene blue follows, 50% of the living healthy cells are intensely stained, while those treated with tap water remain unstained. Glucose increases the action of distilled water considerably, most cells staining within a few minutes. If methylene blue fails to penetrate a cell, the latter will grow by the addition of salt; but salt has no such effect after staining has taken place. Clearly this is not vital staining; death occurs after the entrance of the dye.

GRAY, P. A rapid technique for the permanent mounting of minute fresh-water organisms. J. Royal Micros. Soc., **52**, 370–373. 1932.

This method insures the certainty of obtaining objects fixed to a certain portion of the slide, avoids distortion, is permanent and quite rapid. It may be employed for Rhizopods, both individual and colonial Flagellates, free and semi-parasitic Ciliates and some larval forms, but not for Rotifers, stalked Ciliates or nematodes.

The following "basal fixative" is recommended: Picric acid, 1 g.; mercuric chloride, 1 g.; 95% alcohol, 10 cc. (1) For protozoa use: 10 drops "basal fixative," 3 drops ether, 2 drops glacial acetic, 5 drops 40% formaldehyde. (2) For heavily cuticularized forms use: 10 drops "basal fixative," 1 drop ether, 4 drops glacial acetic, 5 drops 40% formaldehyde. (3) For delicate larvae use: 10 drops "basal fixative," 2 drops ether, 1 drop glacial acetic, 5 drops 40% formaldehyde.

Rinse slides with acetic acid alcohol, wipe, and smear heavily with Meyer's albumen over an area of 1 cm. Lift the object in a pipette with the least possible quantity of water and place on the albumen patch. Let a large drop of the fixative fall upon the organism, guiding it back into place, if necessary, with a fine glass needle. Remove surplus water with filter paper and let droplet evaporate until outline of object is visible. Draw a circle with diamond around the organism, flood with 70% alcohol for a few seconds. Store the slide in 70% alcohol or proceed with staining. For protozoa and larvae, stain with Ehrlich's hematoxylin until the nuclei show well, rinse in 95% alcohol, flush with 0.25% HCl in alcohol, wash with tap water. Counterstain in 0.5% eosin in 50% alcohol, dehydrate, clear in terpineol and mount in balsam.

GUTSTEIN, M. Zur Theorie der Vitalfärbung. Zts. Ges. Exp. Med., **82**, 479–524. 1932.

The vital staining of yeasts by basic stains depends upon: the relative solubility of the dye in the lipoid solvent and in the cell substances; its reducibility by the plant cell; its precipitability by Cranner's phosphatid, (a water soluble substance isolated from the ectoplasm of plant cells). The following stains are taken up by yeasts in the order given: Victoria blue>fuchsin>safranin>Nile blue.

Both lipoid-soluble and non-soluble acid stains are taken up by yeasts, but by the latter to a lesser degree (i.e., colloidal stains as Congo red, brilliant purpur R., trypan blue, etc.). Acid stains may also be reduced by plant cells. They are not precipitated by the water soluble phosphatid. Thymus cells suspended in physiological salt solution show a behavior similar to that of yeasts towards basic and acid dyes. Acid stains not soluble in lipoids probably penetrate the cells as free acids, which are lipoid-soluble.

The fundamental difference between acid and basic stains in their action on the animal cell is that the latter form a stable union while the former merely enter into solution in the cell substance. An hypothesis is offered that the accumulation of acid stains by certain animal cells depends upon a difference in H-ion concentration on the two sides of the cell membrane.

HUNTOON, F. M. Glycerin as an adjuvant to bacterial dyes. Am. J. Clin. Path., **1**, 317–319. 1931.

Glycerin is recommended as a substance tending to stabilize staining solutions, influencing the staining and clearing the microscopic picture. For Gram's stain mix 15 parts of 3% crystal violet in 95% alcohol with 85 parts of 30% aqueous glycerin. Counterstains: (A) Mix 10 cc. of carbol fuchsin with 100 cc. of a 25% aqueous glycerin. (B) Shake up 2 g. of Bismarck brown in 100 cc. of water and filter. Add 30 to 40 cc. of glycerin and mix. The usual Gram's iodine is used. For decolorization use 75 parts alcohol to 25 parts acetone.

For staining of tubercle organism, the above described glycerin crystal violet may be used with Bismarck brown as counterstain; or one may employ glycerin carbol fuchsin, prepared as follows: 5% phenol, 75 cc.; glycerin, 25 cc.; saturated solution of basic fuchsin in 95% alcohol, 10 cc. This combination stains as well as the usual carbol fuchsin, but does not tend to precipitate either in the bottle or on the slide even if boiled.

For a modified Loeffler's methylene blue use 25% aqueous glycerin, 100 cc.; 1% solution of sodium hydrate, 1 cc.; saturated solution of methylene blue in 95% alcohol, 10 cc. This solution gives a sharper picture than the usual. For methylene blue as counterstain to the carbol fuchsin in acid-fast staining, use 2 cc. of saturated alcoholic methylene blue to 100 cc. of a 25% aqueous glycerin.

KOSTOWIECKI, M. Über die Anwendung von Anilinblau und Orange G zur färberischen Darstellung der skelettbildenden Gewebe (Modifikation der Mallory-Heidenhainschen Bindegewebsfärbung). Zts. Wis. Mikr., **49**, 337–340. 1932.

The method may be used on mature animals, but has been developed especially for young embryos before the development of connective tissue fibers, where the Mallory-Heidenhain procedure fails to give sufficient differentiation. Sublimate fixatives are particularly recommended, but other fluids may be used if pretreatment with 3% sublimate precedes staining.

Dissolve 0.2 g. of orange G and 0.06 g. of anilin blue (source of stains not mentioned) in 100 cc. of distilled water; boil for 3 minutes, and add to the hot solution 1 g. of phosphomolybdic acid; cool and filter. The stain keeps for 2 months. Paraffin sections stained in toto with paracarmin are run down to distilled water and stained for ½ to 12 hours. Rinse in distilled water, 1 minute in 95% alcohol, pass thru another alcohol bath, and dehydrate. The color of the anilin blue improves if sections are left in xylol for ½ an hour before mounting; if necessary, differentiate in 90% alcohol. Watch particularly for the future skeletal substance.

Results: Precartilage light ultramarine, cartilage Berlin blue, connective tissue fibers dark blue, regions of ossification and bones light marine blue. The cytoplasm of younger embryos is slightly bluish in all tissues except the muscles, which stain orange. In older embryos all cytoplasm is light orange. Cornified epithelia are orange-yellow in early stages and yellow when cornification has completed. Erythrocytes are dark orange, nuclei brick red to red with a violet tinge.

LEVITSKY, G. A. An essay on cytological analysis of the fixing action of the chrom-acetic formalin and the chromic formalin. Trud. Prik. Bot. Gen. i Sel. (Bul. Apl. Bot. Gen. & Plant Breeding), **27**, 176–185. 1931.

The action of separate components of Navashin's original formula designated "10–4–1" (chromic acid 1%, 10 cc.; formalin, commercial, 4 cc.; glacial acetic, 1 cc.); and various combinations of these were studied on root tips of *Vicia faba*. By staining with iron hematoxylin the following results were obtained: 1) With formalin alone, the chromosomes are quickly and completely decolorized during differentiation. The nucleoli remain coal black, the cytoplasm is clear. The stain fades out irregularly. 2) With acetic acid, the nucleoli are decolorized first, while the chromosomes and the network of metabolic nuclei still remain black. 3) Chromic acid occupies an intermediate position, the chromosomes beginning to decolorize first and then the nucleoli. 4) With formalin and chromic acid, the nucleoli are black, even after the chromosomes are decolorized in the greater part of the plates. 5) Chromic, with acetic acid, gives results similar to chromic acid alone. 6) Formalin, with acetic, is nearly the same as acetic alone, and shows pictures very similar to those obtained with chrom-acetic-formalin.

Thus the action of a compound fixative such as the "10–4–1," may be very well inferred from the separate action of its components. If formalin is completely removed and fixation is effected with 1% chromic acid, the metaphase chromosomes are thick—they appear in close contact, or even fused. The proximal constructions of headed chromosomes are scarcely differentiated. After differentiation they lose the color uniformly, while following "10–4–1" the peripheral parts retain the stain, in consequence of which optical sections show "rings" and "spectacles." After fixing with a large proportion of formalin, the chromosomes of *Vicia faba* fade very promptly and one succeeds in staining them only in the presence of a dark cytoplasm. They appear to be split into two corroded and knotted structures. The proximal heads are separated by achromatic constrictions. By increasing the chromic acid, the chromosomes retain the stain more strongly and the picture is similar to the above, except that the cytoplasm is clear. The combination of 10% formalin and 1% chromic acid is best suited for *V. faba*. By using the fixing fluids containing 1 to 2 parts formalin to 8 to 9 parts chromic acid, the thickness of chromosomes appears quite different, due to the action of iron alum. The fading does not proceed equally thruout the body of the chromosome, but begins at the periphery and then goes to the inner part. In several cases a slightly yellow stained peripheral coat was obtained with a black axial thread within it.

One may conclude that the differences in the thickness of chromosomes shown by the "Chondriosomal" (Levitsky's 10% formalin with 1% chromic acid) or by the "Nuclear" fixative, depend on the staining of either the axial parts of the chromosome or the latter together with its coat layers.

The nucleolus fades very rapidly after the "10–4–1" fixation when the peripheral layer of the chromosomes shows a dense staining. It retains hematoxylin steadily after formalin fixation, while the peripheral layers decolorize.

MOORE, E. J. The use of phenosafranin for staining fungi on culture media or in host tissue. Science, **77**, 23–24. 1933.

Mangin (1890) proposed phenosafranin as a differential stain for pectose and lignin. It proves useful in mycological studies for both fresh and preserved material and in the examination of bacterial colonies on agar. Satory's formula, as given by Linder, is recommended: Carbolic acid crystals 20 g., lactic acid syrup 20 g., glycerin 40 g., distilled water 20 g., phenosafranin 0.5 g., or less. Mordant fixed sections with 2% iron alum for 2 hours. Destain with 0.5% alum dissolved in 0.5% HCl, or with alcohol. Intensify the stain with 1% ammonium hydroxide.

The host tissue destains quicker than the parasite, so by proper control a differential stain is obtained. Sections of Johnson's grass leaves infected with a rust, stained for 20 minutes in phenosafranin and then destained for 10 minutes in acid alum, show a good differential stain.

In the case of mycelia within woody tissue the contrast is not so pronounced.

This stain is particularly adaptable to the study of bacterial and fungal colonies on an agar substratum. If these colonies are treated with phenosafranin the medium does not absorb the stain as readily as the cells, so that the latter stand out intensely stained against a lighter background, no destaining being necessary.

SEKI, MASAJI. The physical chemistry of histological staining. I. Preliminary electrical charge of dyes and its role in the dyeing of SiO_2. Folia Anatomica Japonica, **10**, 621–34. 1932.

Cataphoretic studies of 31 acid and 20 basic dyes in agar gel at pH 7 show that all basic dyes migrate to the cathode and all acid dyes to the anode. Negatively charged SiO_2 is dyed, at pH 7, by all the basic dyes used, but by none of the acid ones.—*A. E. Stearn.*

SEKI, MASAJI. The physical chemistry of histological staining. II. Substantive dyeing of fixed histological preparations. Folia Anatomica Japonica, **10**, 635–54. 1932.

In histological work the action of basic dyes is entirely different from that of acidic ones. Most preparations are of tissue on the alkaline side of the isoelectric points of the constituents, and basic dyes stain by a "chemical adsorption," a mechanism in which size of dye molecule, speed of diffusion, and tendency to form an insoluble precipitate with the substrate are unimportant factors. As the pH increases, dyeing becomes more intense, and the reverse is true with decrease of pH.

Acid dyes, having a charge similar to that of the substrate constituents, cannot dye in this way. They color merely by "infiltration," and are easily washed out with water. With such a mechanism molecular size with proportionate speed of diffusability are important, as is also a tendency "to become insoluble." Thus, dyes of low molecular size and high diffusibility easily penetrate all parts of tissue, whereas large dye molecules can penetrate only tissues with wide "pores." A greater "adsorbability" is assigned to the large molecules, for no specified reason, so that in regions which both types of dye molecule would reach by the diffusion process, the smaller molecule would be driven out in competition with the larger, and would concentrate in tissues impenetrable by the other. Thus a differential staining is obtained. It is pointed out that change in pH affects staining by acid dyes qualitatively in just the opposite way from staining by basic dyes, but this point is not explained.—*A. E. Stearn.*

SHAEFFER, ALICE B. and FULTON, MACDONALD. A simplified method of staining endospores. Science, **77**, 194. 1933.

A comparison of various methods of staining bacterial endospores has been made and best results have been obtained with that of Wirtz (Centbl. Bakt., I Abt., Orig., **46**, 727, 1908). This method, however, calls for fixation of the smears in osmic acid—a step which the authors find can be omitted.

Films of bacteria, made in the usual manner, are fixed by flaming three times. Flood with 5% aqueous malachite green and heat to steaming 3 or 4 times within ½ minute. Wash off the excess stain under the tap for about ½ minute. Apply a 0.5% aqueous safranin solution for ½ minute. Wash, blot, dry, and examine. Spores are stained green, cells red. There is no blending of the colors.

TILGHMAN, R. C. and LEE, F. C. The vital staining of rabbit carcinoma. Bul. Johns Hop. Hosp., **49**, 360–387. 1931.

Rabbits with experimental carcinoma of the testes received intravenous injections of 1% aqu. trypan blue, alone or followed by carmin. The carmin solution employed was 5% carmin rubrum optimum (Grübler) in a saturated solution of lithium carbonate, heated on a water bath for 15 minutes and filtered. At autopsy, the liver and spleen were perhaps stained most deeply; the kidneys stained differently in various rabbits.

The cancers of the rabbits which received injections of both dyes showed no single typical picture, yet there were features common to all. In the gross, none showed a solid and entirely purple coloration. Instead, all showed separate and distinct blue and purple areas diffusely mingled in irregular patterns in the various tumors.

Microscopic examination of Bouin or formalin-fixed material showed that in animals injected with trypan blue alone, the dye was accumulated primarily in the phagocytic wandering mononuclear cells, which were situated in great numbers in the stroma surrounding the tumor nodule. In the animals receiving both carmin and trypan blue, the oldest carcinoma areas stained predominantly blue, the youngest red; while a mixture of these two colors was seen in the regions of intermediate cancer growth. In the phagocytic cells, granules of either or more often of both dyes were observed; but cells were never seen in which one vacuole contained particles of both dyes.